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Fungal Morphogenetic Pathways Are Required for the Hallmark Inflammatory Response during *Candida albicans* Vaginitis

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Vulvovaginal candidiasis, caused primarily by *Candida albicans*, presents significant health issues for women of childbearing age. As a polymorphic fungus, the ability of *C. albicans* to switch between yeast and hyphal morphologies is considered its central virulence attribute. Armed with new criteria for defining vaginitis immunopathology, the purpose of this study was to determine whether the yeast-to-hypha transition is required for the hallmark inflammatory responses previously characterized during murine vaginitis. Kinetic analyses of vaginal infection with *C. albicans* in C57BL/6 mice demonstrated that fungal burdens remained constant throughout the observation period, while polymorphonuclear leukocyte (PMN), S100A8, and interleukin-1β levels obtained from vaginal lavage fluid increased by day 3 onward. Lactate dehydrogenase activity was also positively correlated with increased effectors of innate immunity. Additionally, immunodepletion of neutrophils in infected mice confirmed a nonprotective role for PMNs during vaginitis. Determination of the importance of fungal morphogenesis during vaginitis was addressed with a two-pronged approach. Intravaginal inoculation of mice with *C. albicans* strains deleted for key transcriptional regulators (*bcr1*Δ/Δ, *efg1*Δ/Δ, *cph1*Δ/Δ, and *efg1*Δ/Δ *cph1*Δ/Δ) controlling the yeast-to-hypha switch revealed a crucial role for morphogenetic signaling through the Efg1 and, to a lesser extent, the Bcr1 pathways in contributing to vaginitis immunopathology. Furthermore, overexpression of transcription factors *NRG1* and *UME6*, to maintain yeast and hyphal morphologies, respectively, confirmed the importance of morphogenesis in generating innate immune responses *in vivo*. These results highlight the yeast-to-hypha switch and the associated morphogenetic response as important virulence components for the immunopathogenesis of *Candida* vaginitis, with implications for transition from benign colonization to symptomatic infection.

Candida albicans, an opportunistic polymorphic fungal species, is the leading cause of vulvovaginal candidiasis (VVC) and presents major quality-of-life issues for women worldwide (1). VVC is characterized by itching, burning, and redness of the vulva and vaginal mucosa, often accompanied by a thick white vaginal discharge. It is estimated that 75% of all women will experience an episode of VVC at least once in their lifetime. Major risk factors for development of acute vaginitis include the use of high-estrogen oral contraceptives, hormone replacement therapy, and uncontrolled diabetes mellitus (1). A smaller subset of women (5 to 8%) experience recurrent VVC (RVVC), defined as having 3 or more major vaginitis incidents per year. These chronic bouts of vaginal inflammation are idiopathic in nature, having no clinically recognizable predisposition for infection (2).

Despite its widespread prevalence, relatively little is known about the protective immune mechanisms involved in defense against vaginal candidiasis. It was long believed that women with RVVC harbored defects in systemic or local cell-mediated immunity, as susceptibility to other forms of mucocutaneous candidiasis had been shown to be T-cell dependent (3, 4). However, several cross-sectional clinical studies and supporting data from animal models have demonstrated that adaptive immunity seems to play no major role in protection from disease (5, 6). A paramount study conducted by Fidel et al. using a live intravaginal challenge model in women volunteers led to a paradigm shift in the current philosophy of vaginitis pathogenesis (7). Findings revealed that symptomatic women developed high levels of polymorphonuclear leukocyte (PMN) infiltrate in the vaginal lumen despite having colonization levels similar to those of asymptomatic women. Therefore, it was concluded that PMN migration into the vagina not only was nonprotective but actually appeared to contribute to symptomatology. Those studies also led to the hypothesis that the immunopathogenesis of vaginitis in women with RVVC is virtually the same as that in women with acute VVC but at a higher level of sensitivity for the pathological response.

In an effort to dissect the mechanisms leading to vaginal PMN recruitment during infection, studies using a murine vaginitis model conducted by Yano et al. revealed that the calcium binding proteins S100A8 and S100A9 were highly induced in the vaginal epithelium upon infection (8). Aside from calcium binding activity, the S100A8/9 complex also has an important role in innate immunity, acting as host-derived danger signals during infection to recruit PMNs to active sites of infection (9). Along with other self-recognized factors, such as heat shock proteins and high-mobility-group box 1 (HMGB1), these signals are collectively termed "alarmins" (10). Although the S100A8/9 complex undoubtedly has a role during vaginitis immunopathogenesis, studies using S100A9 knockout mice (deficient in both functional S100A8 and S100A9) and eukaryote-derived recombinant S100 proteins re-

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TABLE 1 Strains used in this study

Strain	Designation	Genotype	Reference
DAY185	WT	*ura3*Δ::*imm434/ura3*Δ::*imm434 HIS1*::*his1*::*hisG/his1*::*hisG*::*URA3*::*arg4*::*hisG/arg4*	51
CJN702	*bcr1*Δ/Δ	*ura3*Δ::*imm434/ura3*Δ::*imm434 arg4*::*hisG/arg4*::*hisG his1*::*hisG*::*pHIS1/his1*::*hisG bcr1*::*ARG4/bcr1*::*URA3*	28
CJN698	*bcr1*Δ/Δ+*BCR1*	*ura3*Δ::*imm434/ura3*Δ::*imm434 arg4*::*hisG/arg4*::*hisG his1*::*hisG*::*pHIS1-BCR1/his1*::*hisG bcr1*::*ARG4/bcr1*::*URA3*	28
HLC52	*efg1*Δ/Δ	*ura3*Δ::*imm434/ura3*Δ::*imm434 efg1*::*hisG/efg1*::*hisG-URA3-hisG*	30
HLC74	*efg1*Δ/Δ+*EFG1*	*ura3*Δ::*imm434/ura3*Δ::*imm434 efg1*::*hisG/efg1*::*hisG LEU2/LEU2*::*pEFG1-URA3-LEU2*	30
JKC19	*cph1*Δ/Δ	*ura3*Δ::*imm434/ura3*Δ::*imm434 cph1*::*hisG/cph1*::*hisG-URA3-hisG*	30
HLC54	*efg1*Δ/Δ *cph1*Δ/Δ	*ura3*Δ::*imm434/ura3*Δ::*imm434 cph1*::*hisG/cph1*::*hisG efg1*::*hisG/efg1*::*hisG-URA3-hisG*	30
THE21	THE21	*ade2*::*hisG/ade2*::*hisG ura3*::*imm434/ura3*::*imm434 ENO1/eno1*::*ENO1-tetR-ScHAP4AD-3XHA-ADE2*	19
TT21	TT21	*ade2*::*hisG/ade2*::*hisG ura3*::*imm434/ura3*::*imm434*::*URA3-tetO-ENO1/eno1*::*ENO1-tetR-ScHAP4AD-3XHA-ADE2*	20
TNRG1	TNRG1	*ade2*::*hisG/ade2*::*hisG ura3*::*imm434/ura3*::*imm434*::*URA3-tet-O-NRG1 ENO1/eno1*::*ENO1-tetR-ScHAP4AD-3XHA-ADE2*	This study
TUME6	TUME6	*ade2*::*hisG/ade2*::*hisG ura3*::*imm434/ura3*::*imm43*::*URA3-tetO-UME6 ENO1/eno1*::*ENO1-tetR-ScHAP4AD-3XHA-ADE2*	20

vealed their "sufficient but not necessary" role as PMN chemotactic factors in the vaginal epithelium (52). Other compensatory host factors are also likely involved in the inflammatory response during vaginitis, including the innate cytokine interleukin-1β (IL-1β). Indeed, elevated levels of IL-1β have been demonstrated during symptomatic infection (clinically and in the mouse model), although IL-1β's role in vaginitis immunopathology remains unknown (11, 12).

Because innate immune function has been recognized as being highly relevant to the immunopathogenesis of *Candida* vaginitis, dissection of the fungal virulence factors responsible for initiating PMN recruitment to the vaginal epithelium is critical for a fuller comprehension of disease pathology. The ability to switch between yeast and hyphal forms has long been considered the major virulence factor of *C. albicans*. The yeast-to-hypha switch is controlled by a complex series of environmental sensors coupled to regulatory transcription factors that encode hypha formation, adhesin production, secreted virulence factors (e.g., secreted aspartyl proteinases), stress response elements, and biofilm-associated factors (13). Despite knowing that these transcription factors are presumably activated during vaginal infection (by evidence of biofilm formation on the vaginal epithelium), there exists a significant gap in knowledge as to the effects of fungal morphogenesis on the immunopathogenesis of vaginitis (14). While *in vitro* epithelial infection models have demonstrated yeast- and hypha-specific immune responses during challenge with *C. albicans*, comprehensive *in vivo* studies have been sorely lacking (15). In fact, the only study addressing this issue was conducted by Sobel et al., using a spontaneous mutant of *C. albicans* that failed to form hyphae (16). Nevertheless, a precedent for morphological transitions being important for pathogenesis was reported.

Therefore, the aim of this work was to determine whether fungal morphogenetic regulation contributes to vaginitis immunopathology by using *C. albicans* strains defective in key transcriptional regulators involved in the yeast-to-hypha switch. We also propose novel vaginitis biomarkers and signaling pathways indicative of broader mucosal defense strategies against *C. albicans*.

MATERIALS AND METHODS

Ethics statement. The animals used in this study were housed in AAALAC-approved facilities located at the LSU Health Sciences Center (LSUHSC) in the School of Dentistry. The LSUHSC Animal Care and Use Committee approved all animals and protocols. Mice were given standard rodent chow and water *ad libitum*. Mice were monitored daily for signs of distress, including noticeable weight loss and lethargy.

***C. albicans* strains.** A comprehensive list of all *C. albicans* strains used in these studies can be found in Table 1. The wild-type (WT) strain used for these studies was DAY185. Strain DAY185 is a complemented prototroph derived from a triple auxotrophic strain (BWP17; parent, SC5314), which is frequently used to produce knockout and complemented strains of *C. albicans*. Strains HLC52 (*efg1*Δ/Δ), HLC74 (*efg1*Δ/Δ+*EFG1*), JKC19 (*cph1*Δ/Δ), and HLC54 (*efg1*Δ/Δ *cph1*Δ/Δ) were kindly provided by G. R. Fink (Whitehead Institute, Cambridge, MA, USA). Strains CJN702 (*bcr1*Δ/Δ) and CJN698 (*bcr1*Δ/Δ+*BCR1*) were a gift from A. P. Mitchell (Carnegie Mellon University, Pittsburgh, PA, USA). Strains containing NRG1 (TNRG1), UME6 (TUME6), and an empty vector (TT21) under the control of a tetracycline-regulatable promoter were also constructed. They behave similarly to those described previously by Kadosh et al. and Saville et al., but our strategy enabled the construction of an isogenic control strain (TT21) as well as full restoration of the *URA3* locus (17, 18). The strains were generated as follows. Strain THE1 was kindly provided by H. Nakayama and M. Arisawa (Nippon Roche) (19). pKEtetO4 was previously described and is based on plasmid pLUX (20). pKEtetO4 harbors the *tetO* promoter and the 3′ untranslated region (UTR) of the *C. albicans ADH1* gene, separated by a multiple-cloning site (MCS). The *NRG1* open reading frame (ORF) was amplified with primer set NRG1ORFF-SalI (5′-TCAGTCGACATCATTATGCTTTATCAACAATC-3′) and NRG1ORFR-MluI (5′-TCAACGCGTTTGACCACATCTATACTAGGC-3′) and cloned downstream of the *tetO* promoter between the SalI and MluI restriction sites (underlined in sequences) of pKEtetO4. The expression construct was linearized with NheI prior to transformation into *ura3*Δ/Δ strain THE1, using the lithium acetate method to yield strain TNRG1 (21). Correct integration of this vector fully restores endogenous *URA3* and adjacent *IRO1* loci. This was confirmed by PCR using primers LUXINTF (5′-CTGACCTTTAGTCTTTCCTGC-3′) and LUXINTR (5′-CAGTAGTACTTGTTGTTGTATCG-3′). An isogenic control strain (TT21), made by transforming the pKEtetO4 vector alone into THE1, was previously described, as was the isogenic TUME6 strain (20).

Phenotypes were confirmed by microscopy after inoculating strains into yeast extract-peptone-dextrose (YPD) and RPMI medium with or without 20 μg/ml doxycycline and incubating them at 30°C or 37°C overnight with shaking at 200 rpm (see Fig. S1 in the supplemental material). Growth curves were also performed to compare TT21, TNRG1, and TUME6 by measuring the optical density at 600 nm (OD_{600}) at hourly intervals. No significant growth defects among strains were noted (see Fig. S2 in the supplemental material).

Microorganism growth. *C. albicans* was maintained as a glycerol stock stored at −80°C. A small amount of stock was spread onto YPD and incubated at 30°C for 48 h to generate isolated colonies. A single colony was transferred into 10 ml of liquid YPD and incubated at 30°C with shaking at 200 rpm for 18 h prior to intravaginal inoculation. Use of the TUME6 strain required the addition of 20 μg/ml doxycycline to solid and liquid YPD media to maintain this organism as a yeast for accurate counting prior to intravaginal inoculation.

Murine model of *Candida* vaginitis. The murine model of *Candida* vaginitis has been reported extensively in the literature and was described previously (22). C57BL/6 mice were purchased from Jackson Laboratories and housed in isolator cages mounted onto ventilated racks. Mice were administered 0.1 mg of estrogen (β-estradiol 17-valerate; Sigma) dissolved in 0.1 ml sesame oil subcutaneously 72 h prior to inoculation with *C. albicans*. Estrogen injections were administered weekly thereafter if required. Stationary-phase cultures of *C. albicans* isolates were washed three times in sterile, endotoxin-free phosphate-buffered saline (PBS) and resuspended in a 0.2× volume of PBS. Cell suspensions were diluted, counted on a Neubauer hemocytometer, and adjusted to 2.5×10^8 CFU/ml in sterile PBS. Estrogen-treated mice were intravaginally inoculated with 20 μl of the standardized blastoconidial cell suspension, generating an inoculum size of 5×10^6 blastoconidia. Naive controls were inoculated with 20 μl of sterile PBS. All animal experiments were conducted in duplicate.

Vaginal lavage. Groups of mice ($n = 4$ or 5) underwent vaginal lavage immediately after sacrifice with 0.1 ml of sterile PBS containing protease inhibitors (cOmplete, EDTA free; Roche) at day 1, 3, or 7 postinoculation. In longitudinal experiments, mice were briefly anesthetized by isoflurane inhalation, lavaged at day 3 postinoculation with protease inhibitor-free PBS, returned to their cage, and again lavaged at day 7 immediately after sacrifice. Longitudinal samples were immediately spiked with 1 μl of 100× protease inhibitor cocktail (cOmplete) postlavage, so as to avoid any potential unwanted effects of protease inhibitor administration during infection. Recovered lavage fluids were kept on ice during processing. Aliquots were removed to determine fungal burden and for PMN enumeration. The remaining lavage fluid was centrifuged at 3,600 rpm for 3 min at 4°C to remove cellular debris, filtered through a 0.2-μm syringe filter, and stored at −80°C until needed.

Neutrophil depletion. In some experiments, mice were injected intraperitoneally (i.p.) with either 200 μg rat anti-mouse Ly6G or rat IgG2A isotype control antibodies (Bio-X-Cell) in 0.1 ml sterile PBS to systemically deplete PMNs, as described previously (23). Mice were injected with antibodies 1 day prior to intravaginal inoculation with *C. albicans* and every 3 days thereafter. Neutrophil depletion was confirmed by counting PMNs in vaginal lavage fluid (see details below).

Fungal burden. Lavage fluid was serially diluted 10-fold in sterile PBS and plated onto YPD agar containing 50 μg/ml chloramphenicol to inhibit bacterial overgrowth, using the drop-plate method as described previously (24). Plates were allowed to dry and then incubated for 24 h at 37°C, and the resulting colonies were enumerated. CFU/ml values per group are reported as medians.

PMN quantification. Lavage fluid (10 μl) was smeared onto Tissue Path Superfrost Plus Gold slides (Fisher Scientific), allowed to air dry, fixed with CytoPrep spray fixative (Fisher Scientific), and stored at room temperature. Slides were then stained by using the Papanicolaou technique ("Pap smear"). PMNs were identified by their morphology, staining appearance, and characteristic trilobed nuclei. For each smear, PMNs were manually counted in five nonadjacent fields by standard light microscopy using a 40× objective. Smears containing an average of 50 PMNs per field or greater were considered high-PMN responders. PMN counts were averaged per field. Values are reported as the mean PMN count per group ± the standard error of the mean (SEM).

Enzyme-linked immunosorbent assay for S100A8 and IL-1β. Lavage fluid was diluted appropriately (typically 1:10 to 1:1,000) and analyzed for S100A8 protein by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Rndsystems, Inc.) according to the manufacturer's protocol. This kit has a range of detection from 31.25 to 2,000 pg/ml. Lavage fluid was also analyzed for IL-1β by using the commercial Ready Set Go ELISA kit (Ebioscience) according to the manufacturer's protocol. Samples were typically diluted 1:20 prior to analysis. Using this kit, IL-1β can be reliably measured at a range of 8 to 2,000 pg/ml, with a sensitivity of 8 pg/ml. All samples were measured in duplicate and averaged. Values are reported as the mean concentration per group in pg/ml ± SEM.

Lactate dehydrogenase activity assay. Lactate dehydrogenase (LDH) activity was measured in lavage fluid by diluting samples 1:100 in PBS and then following the outlined protocol for the commercially available CytoTox 96 nonradioactive cytotoxicity assay kit (Promega). Positive controls containing purified LDH and negative controls (PBS) were also tested alongside samples to validate the assay. Data are reported as the OD_{492}. Results were averaged per animal group and are reported as the means ± SEM.

Statistical analyses. All experiments were conducted using groups of mice ($n = 4$ or 5) and repeated in duplicate as determined by power analyses. All data were plotted and analyzed for statistical significance by using GraphPad Prism software. Data for most studies were compared by using one-way analysis of variance (ANOVA) and Dunnett's posttest. For the PMN depletion studies, an unpaired Student *t* test was used to compare groups. A paired Student *t* test was also used to compare values for day 3 to those for day 7 within infection groups for the longitudinal study. To determine the correlation significance of S100A8 and IL-1β quantification with PMN levels, a linear regression curve and Spearman's correlation test were used. Graphs were annotated to denote significance levels.

RESULTS

Kinetics of murine *Candida* vaginitis. In order to determine the kinetics of vaginitis progression, groups of C57BL/6 mice were estrogen treated, inoculated with *C. albicans* strain DAY185, and sacrificed at days 1, 3, and 7 postinoculation. Fungal burdens obtained from the vaginal lavage fluid demonstrated a slight but nonsignificant decline from day 1 through day 7, with an average burden of approximately 1×10^5 CFU/ml (Fig. 1A). Naive mice harbored no *Candida*, as expected. PMN quantification demonstrated that PMNs were nearly absent in naive mice and at the day 1 time point but increased significantly from day 3 onward (Fig. 1B). Levels of S100A8 and the innate proinflammatory cytokine interleukin-1β (IL-1β) were also measured in the vaginal lavage fluid. Production of both S100A8 (Fig. 1C) and IL-1β (Fig. 1D) followed expression kinetics similar to those of PMN migration into the vaginal lumen: levels were reduced in naive mice and at day 1 postinoculation but increased significantly and plateaued at day 3. In order to determine whether this seemingly rampant vaginal inflammation was associated with tissue damage, LDH activity (a known marker of cellular damage) was measured in the lavage fluid (25). LDH activity peaked by day 3 postinoculation and remained increased at day 7 (Fig. 1E), while levels were nearly undetectable at day 1 and in naive mice. Lastly, Spearman's correlation test was used to determine that both IL-1β (Fig. 1F) and S100A8 (data not shown) levels were significantly correlated with PMN levels recovered from the vaginal lavage fluid. Because all of the responses identified in Fig. 1 showed significant peaks at day 3 postinoculation but no significant change by day 7, subsequent studies assessed various disease parameters solely at day 3 unless otherwise noted.

***In vivo* effects of neutrophil depletion during vaginitis.** In order to determine the contributions of PMNs to other pathological parameters, groups of mice were administered rat anti-mouse

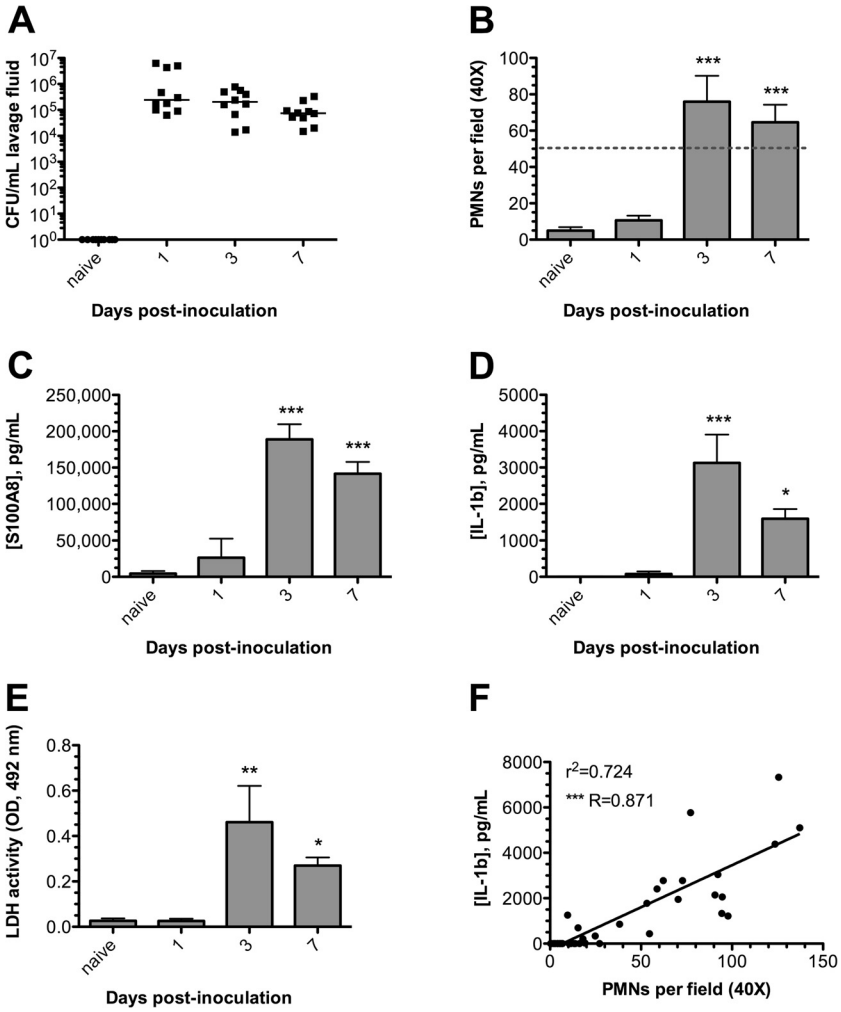

FIG 1 Kinetics of fungal burdens and immunopathological markers during murine vaginitis. Mice were estrogen treated, inoculated with 5×10^6 CFU of *C. albicans*, and sacrificed at specified time points and underwent vaginal lavage. Naive, estrogen-treated mice served as controls. (A to E) Lavage fluids were assessed for fungal burdens by standard microbiological plating (medians) (A), polymorphonuclear leukocytes (PMN) by Pap staining (means ± SEM) (the horizontal dashed line denotes the "high-responder" cutoff) (B), S100A8 protein by ELISA (means ± SEM) (C), interleukin-1β (IL-1β) by ELISA (means ± SEM) (D), and tissue damage by lactate dehydrogenase (LDH) activity (means ± SEM) (E). (F) IL-1β and PMN values were tested for correlation by using Spearman's test. All data are cumulative data from two independent experiments ($n = 5$ per group; 10 mice total). Statistical analysis for significance was achieved by using one-way analysis of variance (ANOVA) with Tukey's posttest. Significance is denoted as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Ly6G antibodies to deplete PMNs. Control animals received rat IgG2A isotype control antibodies. In order to confirm that the PMN depletion was successful, PMNs in the vaginal lavage fluid of inoculated mice were enumerated, and a highly significant decrease was noted for the anti-Ly6G-treated animals (Fig. 2B). Depletion of PMNs had no effect on the fungal burden (Fig. 2A), confirming previous hypotheses that during vaginitis, PMNs are unexpectedly pathological and nonprotective (7, 26). Both S100A8 (Fig. 2C) and IL-1β (Fig. 2D) levels were significantly decreased in the lavage fluid of PMN-depleted mice. Interestingly, LDH activity levels were similar between both groups (Fig. 2E). Vaginal neutrophils were reduced by 95% in the PMN-depleted animals, but S100A8 and IL-1β levels were reduced by only 80% (Fig. 2F).

Morphogenesis is required for *Candida* vaginitis inflammation. Because tissue damage required active infection, we sought to determine whether defects in fungal morphogenesis resulted in altered vaginitis immunopathology. To accomplish this task, we first utilized the following series of *C. albicans* mutants (and their reconstituted strains when appropriate) defective in various transcription factors controlling morphogenetic regulation in the mouse model of vaginitis: *bcr1*Δ/Δ, *efg1*Δ/Δ, *cph1*Δ/Δ, and *efg1*Δ/Δ *cph1*/Δ/Δ. Strain DAY185 served as the WT control. Strains with *BCR1* deleted are able to form morphologically normal hyphae but have significantly reduced expression of hyphal adhesins and secreted virulence factors and an impaired capacity to form biofilms (27, 28). Cph1 is a transcription factor regulated through the mitogen-activated proteinase kinase (MAPK) pathway, and strains lacking *CPH1* expression fail to make hyphae under certain laboratory and stress conditions (29). Efg1 signals downstream of the cyclic AMP (cAMP)/protein kinase A (PKA) pathway, and mutants defective in *EFG1* expression are severely attenuated in the yeast-to-hypha transition, even under strong filament-inducing conditions, such as serum (30, 31). The

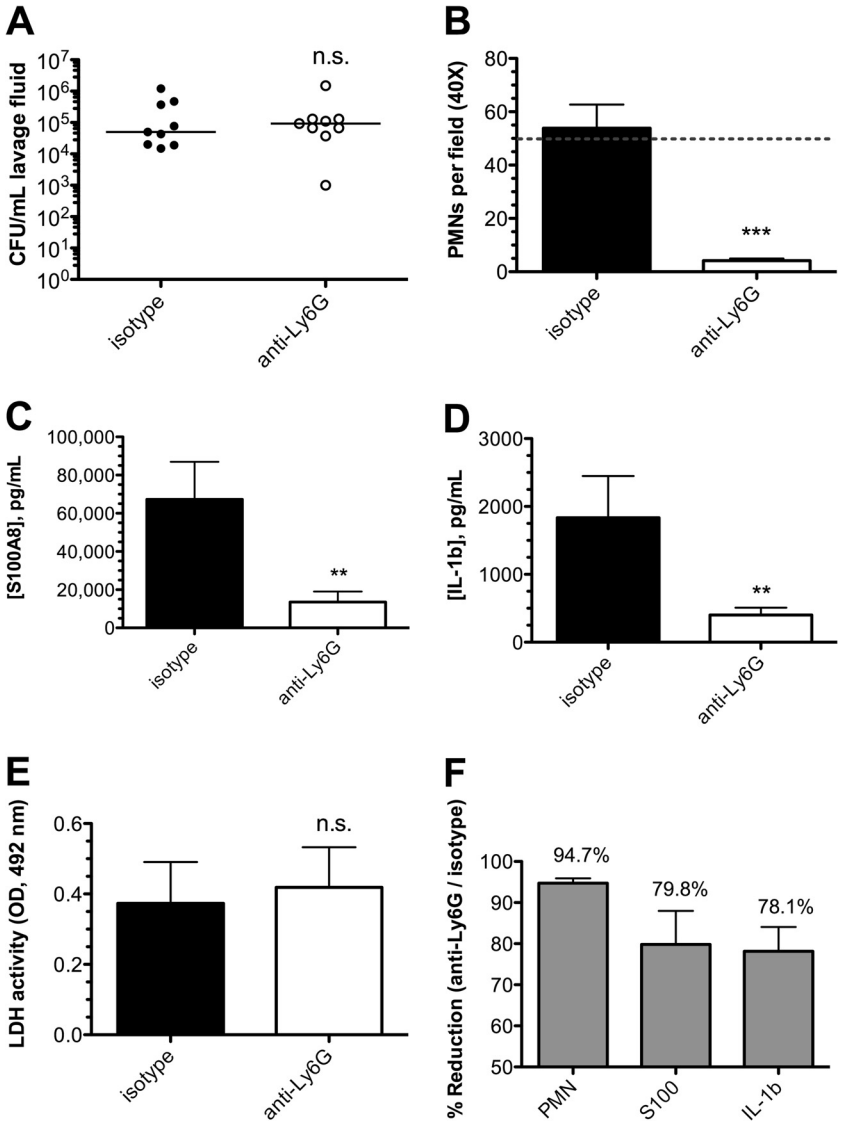

FIG 2 PMNs recruited to the vagina during infection are nonprotective and contribute to pathology but do not cause tissue damage. Mice were intraperitoneally injected with rat anti-mouse Ly6G antibodies prior to infection to deplete PMNs. Control mice were administered an equal amount of rat IgG2A isotype antibodies. (A to E) Vaginal lavage fluids were assessed at day 3 postinoculation for fungal burdens by microbiological plating (medians) (A), PMNs by Pap staining (means ± SEM) (the horizontal dashed line denotes the high-responder cutoff) (B), S100A8 by ELISA (means ± SEM) (C), IL-1β by ELISA (means ± SEM) (D), and tissue damage by LDH assay (means ± SEM) (E). (F) Relative decreases in inflammatory effectors in the anti-Ly6G-treated animals compared to those receiving isotype control antibodies were also calculated. All data are cumulative data from two independent experiments ($n = 5$ per group; 10 mice total). Tests for significance were conducted by using an unpaired Student *t* test. Significance is denoted as follows: n.s., not significant; **, $P < 0.01$; ***, $P < 0.001$.

*efg1*Δ/Δ *cph1*/Δ/Δ double mutant has both transcription factors deleted and exhibits even more severe morphogenesis defects than either single mutant (30).

Fungal burdens were assessed in the vaginal lavage fluid at day 3 postinoculation. Surprisingly, strains defective in the yeast-to-hypha transition (*efg1*Δ/Δ and *efg1*Δ/Δ *cph1*Δ/Δ) counterintuitively demonstrated significant increases in colonization compared to their reconstituted counterparts or the WT control (Fig. 3A). Deletion of *BCR1* or *CPH1* seemed to have no significant effect on fungal burden. Levels of PMNs (Fig. 3B), S100A8 (Fig. 3C), IL-1β (Fig. 3D), and LDH activity (Fig. 3E) in the vaginal lavage fluid were significantly decreased for the *efg1*Δ/Δ and *efg1*Δ/Δ *cph1*Δ/Δ strains compared to the WT despite having significantly higher fungal burdens. Interestingly, the *BCR1* mutation also led to a reduced capacity to elicit an immune response (Fig. 3B to D) despite its ability to form hyphae. Reconstitution of *EFG1* and *BCR1* restored defects comparable to WT levels (*bcr1*Δ/Δ+*BCR1* and *efg1*Δ/Δ+*EFG1*) (Fig. 3). These results indicate that deletion of *CPH1* has little effect on vaginitis immunopathology. Importantly, these experiments also clearly demonstrate that induction of the classic markers of inflammation associated with vaginitis immunopathology require a functional morphogenetic response via the Efg1 and Bcr1 pathways in *C. albicans*.

Morphogenetic override affects vaginitis immunopathology. To determine if the observed results reflected an Efg1-specific function or suggested a more general requirement for regulation

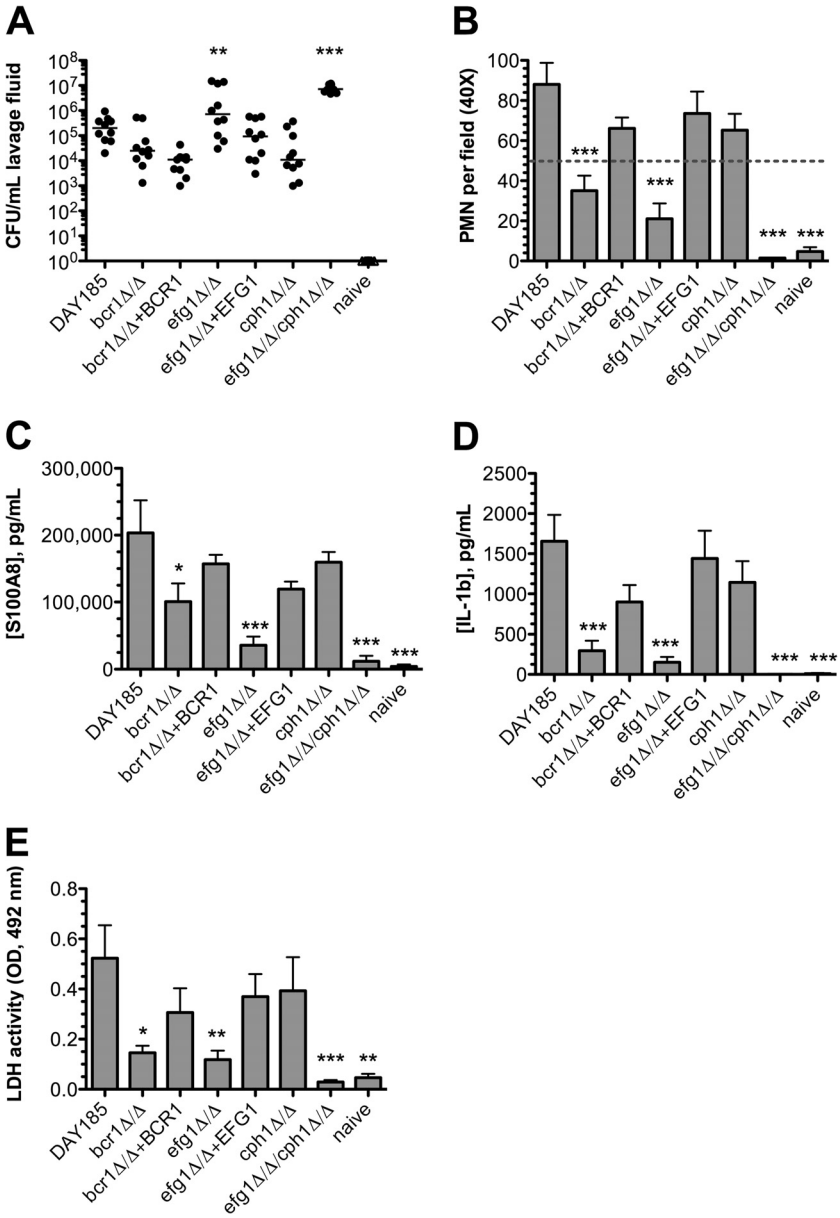

FIG 3 Morphogenetic signaling in *C. albicans* is crucial for the characteristic immunopathology observed during *Candida* vaginitis. Mice were intravaginally inoculated with *C. albicans* strains defective in key transcriptional regulators controlling the yeast-to-hypha switch (and their reconstituted strains when appropriate). The strains used were strain DAY185 and the *bcr1*Δ/Δ, *bcr1*Δ/Δ+*BCR1*, *efg1*Δ/Δ, *efg1*Δ/Δ+*EFG1*, *cph1*Δ/Δ, and *efg1*Δ/Δ *cph1*Δ/Δ strains. Naive, estrogen-treated mice served as controls. Vaginal lavage fluids obtained from mice were analyzed at day 3 postinoculation for fungal burdens (medians) (A), PMN enumeration by microscopy (means ± SEM) (the horizontal dashed line denotes the high-responder cutoff) (B), S100A8 by ELISA (means ± SEM) (C), IL-1β by ELISA (means ± SEM) (D), and tissue damage by LDH assay (means ± SEM) (E). All data are cumulative data from two independent experiments (n = 5 per group; 10 mice total). Statistical testing of significance was achieved by using a one-way ANOVA with Dunnett's posttest. Significance is denoted as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

of *C. albicans* morphogenesis, we used two additional, well-characterized regulators (Nrg1 and Ume6) of the yeast-to-hypha transition in the murine vaginitis model. We also sought to determine whether forcing *C. albicans* to grow constitutively in the hyphal form would exacerbate immunopathology. This complementary approach was achieved by using strains overexpressing *NRG1* or *UME6* under the control of a tetracycline-regulatable promoter to further explore the role of morphogenesis in the immunopathogenesis of vaginitis. Nrg1 (negative regulator of germination) exerts its effects by recruiting the corepressor Tup1 to the promoter regions of hypha-associated genes, thereby blocking their expression (32). Ume6 is required for hypha formation and is coregulated with Nrg1 expression in a negative-feedback system to control the intensity and duration of hyphal extension (33). Therefore, by overexpressing *NRG1* or *UME6* under the control of a tetracycline-regulatable promoter, we were able to dictate the morphological specificity of *C. albicans* as yeast or hypha, respectively (see Fig. S2 in the supplemental material). Mice were inoc-

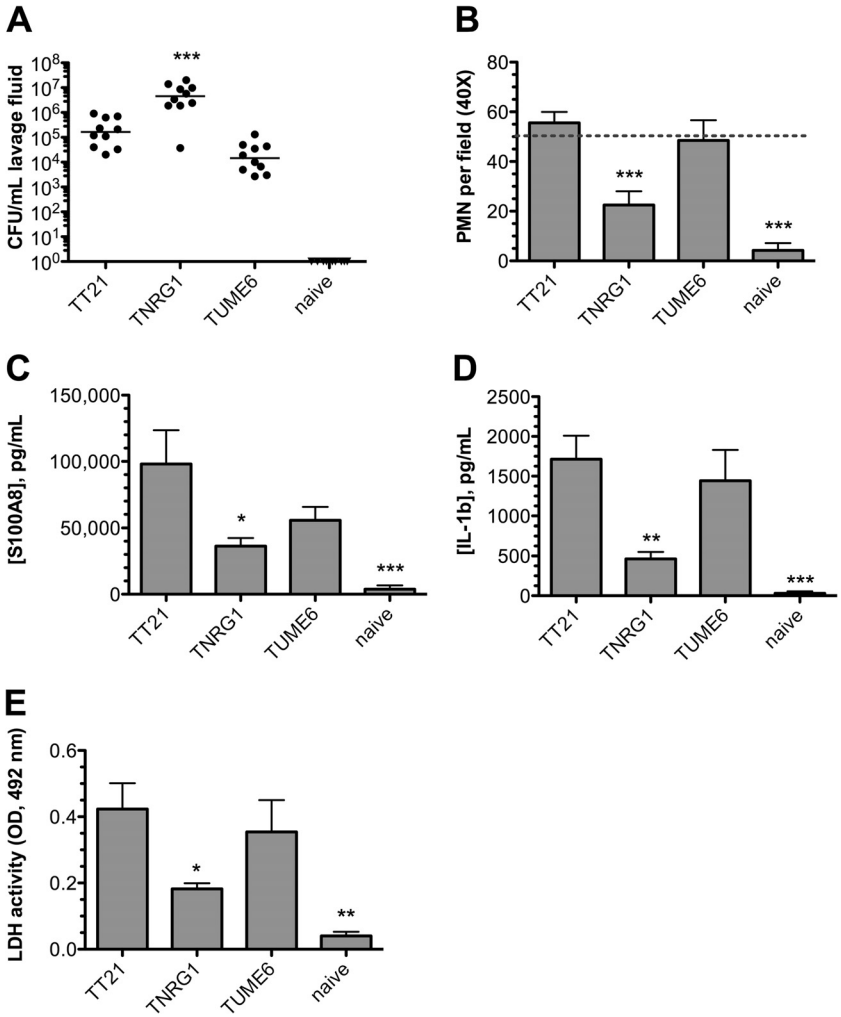

FIG 4 Overexpression of *NRG1* and *UME6* confirms that vaginitis immunopathology is dependent on the yeast-to-hypha transition. Mice were intravaginally inoculated with strains overexpressing transcriptional regulators that force *C. albicans* to remain locked in the yeast (TNRG1) or hyphal (TUME6) form. An isogenic *C. albicans* strain harboring an empty vector under regulation of the *tetO* system was also used as a control (TT21). Vaginal lavage fluids obtained from mice were analyzed at day 3 postinoculation for fungal burdens (medians) (A), PMNs by Pap staining (means ± SEM) (the horizontal dashed line denotes the high-responder cutoff) (B), S100A8 by ELISA (means ± SEM) (C), IL-1β by ELISA (means ± SEM) (D), and tissue damage by LDH assay (means ± SEM) (E). All data are cumulative data from two independent experiments ($n = 5$ per group; 10 mice total). Statistical analysis using one-way ANOVA with Dunnett's posttest was conducted. Significance is denoted as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

ulated intravaginally with TT21 (isogenic control), TNRG1 (yeast locked), or TUME6 (hypha locked), as described above.

Similar to the data obtained for the transcriptional regulator mutants, the TNRG1 strain (yeast locked in the absence of doxycycline) demonstrated significantly higher fungal burdens in the vaginal lavage fluid than did strains TT21 and TUME6, which are able to germinate (Fig. 4A). Despite higher levels of colonization, levels of PMNs (Fig. 4B), S100A8 (Fig. 4C), IL-1β (Fig. 4D), and LDH activity (Fig. 4E) were significantly reduced in the TNRG1 strain compared to the control. Fungal burden and inflammatory mediator levels were similar among control strain TT21 and TUME6. Therefore, using an alternative approach, we have identified that disruption of the yeast-to-hypha transition in *C. albicans* results in reduced immunopathology during vaginitis.

Confirmation of *C. albicans* morphological defects *in vivo*. Morphogenetic phenotypes of the strains used in these studies are classically defined under controlled laboratory settings, but several reports showed variable phenotypes under *in vivo* conditions (34–37). In order to confirm the morphological defect phenotypes *in vivo*, microscopy images were captured from vaginal lavage smears. Mice inoculated with WT strain DAY185 (Fig. 5A); the *bcr1*Δ/Δ (Fig. 5B), *bcr1*Δ/Δ+*BCR1* (Fig. 5C), *efg1*Δ/Δ+*EFG1* (Fig. 5E), and *cph1*Δ/Δ (Fig. 5F) strains; TT21 (Fig. 5H); and TUME6 (Fig. 5J) demonstrated copious amounts of hyphae in the vaginal lavage fluid. As expected, lavage fluids obtained from mice inoculated with the *efg1*Δ/Δ strain (Fig. 5D), the *efg1*Δ/Δ *cph1*Δ/Δ strain (Fig. 5G), or TNRG1 (Fig. 5I) were devoid of hyphae and contained only yeast. Therefore, as predicted, results shown in Fig. 3 and 4 can likely be explained by an inability of *C. albicans* to undergo morphogenetic signaling.

Longitudinal study of immunopathology. In order to determine whether the morphogenetic-dependent immunopathological defects observed at day 3 postinoculation are maintained by day 7, a longitudinal analysis was performed. Only *C. albicans*

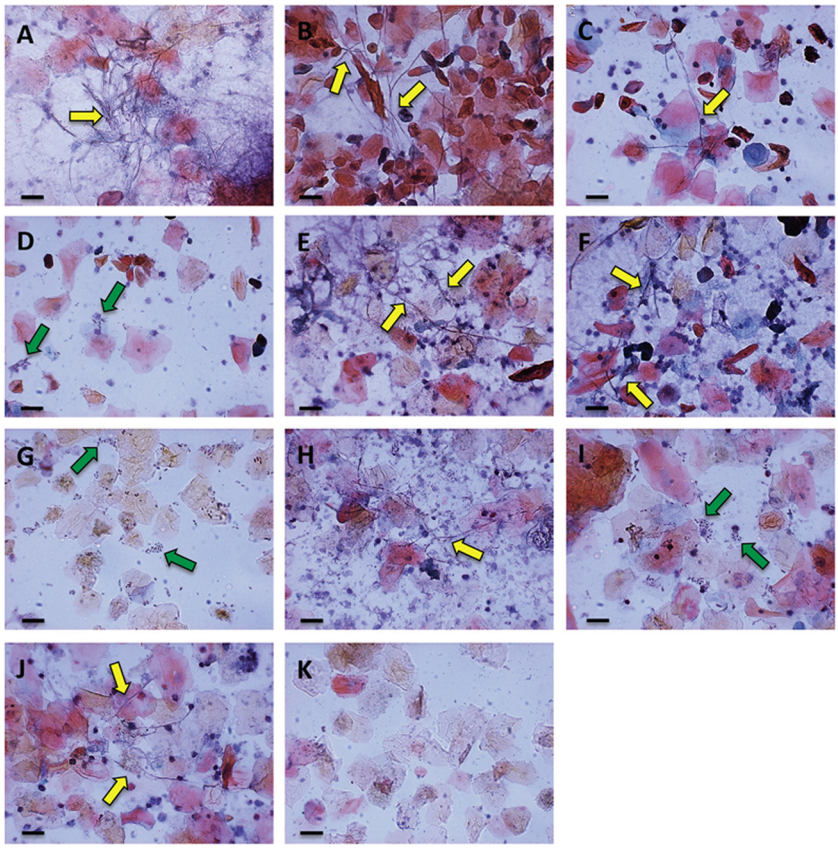

FIG 5 *C. albicans* morphogenesis transcriptional regulator mutants and tetracycline-regulatable strains adopt predicted morphologies *in vivo* during vaginitis. Standard light microscopy using a 40× objective was used to capture images of lavage fluid smears obtained at day 3 postinoculation and stained by using the Pap technique. The various strains depicted are DAY185 (A), the *bcr1*Δ/Δ strain (B), the *bcr1*Δ/Δ+*BCR1* strain (C), the *efg1*Δ/Δ strain (D), the *efg1*Δ/Δ+*EFG1* strain (E), the *cph1*Δ/Δ strain (F), the *efg1*Δ/Δ *cph1*Δ/Δ strain (G), TT21 (H), TNRG1 (I), TUME6 (J), and naive mouse (K). Green arrows denote yeast cells, and yellow arrows denote hypha formation. Bars, 25 μm. Images are representative of each strain.

strains demonstrating early divergent responses were selected for the longitudinal study. As described above, vaginal fungal burdens remained elevated for strains defective in germination (Fig. 6A). Levels of PMNs (Fig. 6B), S100A8 (Fig. 6C), IL-1β (Fig. 6D), and LDH (Fig. 6E) remained suppressed at day 7 during inoculation with the *efg1*Δ/Δ strain, the *efg1*Δ/Δ *cph1*Δ/Δ strain, and TNRG1. Levels of inflammatory mediators measured during inoculation with the *bcr1*Δ/Δ strain were reduced compared to those of WT infection but were generally elevated compared to those of infection with strains unable to undergo morphogenesis (Fig. 6). Overall, there were no significant changes in these inflammatory parameters between day 3 and day 7. These results confirm our previous observation that defects in the ability of *C. albicans* to undergo morphogenesis lead to an inability to generate sufficient signaling of innate immune pathways.

DISCUSSION

Despite decades of research attempting to define a role for adaptive immunity in susceptibility to *Candida* vaginitis, no concrete evidence exists (5). Consequently, the emergence of recent studies has suggested that the inflammatory nature of RVVC/VVC is likely attributable to exuberant activation of host innate immune responses at the vaginal mucosal interface, characterized by the release of inflammatory S100 alarmins and IL-1β (this study) and, ultimately, PMN migration into the vaginal lumen (7, 8). Previously, the gold standard for determining strain-dependent defects of *C. albicans* to cause disease in the animal model of vaginitis relied on quantitative fungal burdens. However, until now, no observable phenotypes had been identified. With characterization of new criteria for measuring symptomatic infection (presence of PMNs, S100A8, and IL-1β), testing of new and previous hypotheses using *C. albicans* mutants is now possible (38). As such, the goal of these studies was to further elucidate the role of fungal morphogenesis in disease pathology.

Kinetics of vaginal infection in C57BL/6 mice closely mirrored those reported by Yano et al. (Fig. 1) (8). It is interesting to note that *C. albicans* begins undergoing the yeast-to-hypha switch within hours after vaginal inoculation, and hyphae can be recovered from the vaginal lavage fluid by day 1 postinoculation (14). However, the immune response does not become fully activated until day 2 postinoculation, suggesting several potential interpretations (8). The simplest explanation is that the activation of epithelial cells required for recruitment of PMNs into the vagina and subsequent expression of high levels of innate cytokines require several days to reach levels detectable by standard laboratory assays. Also, significant invasion of the vaginal epithelium by hyphal forms likely takes time to reach deeper tissue layers, which may lead to robust host responses. An alternative explanation could be

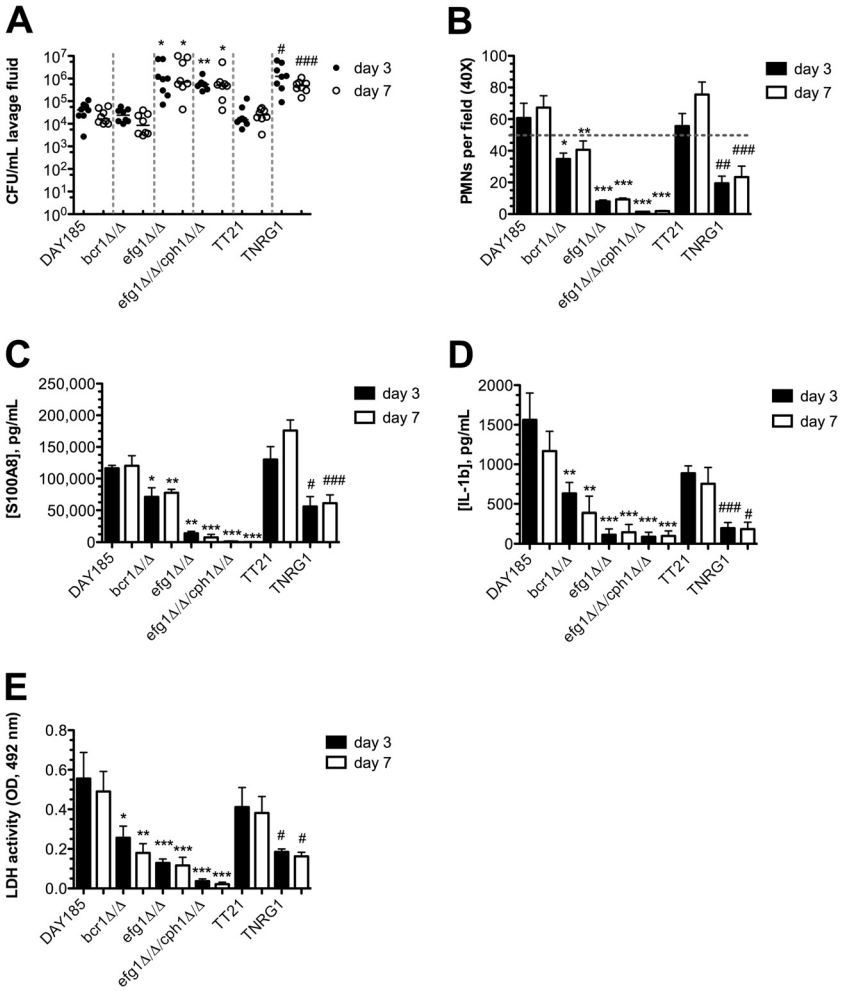

FIG 6 Longitudinal analysis of fungal burdens and inflammatory markers in vaginal lavage fluid. Mice were inoculated with transcriptional regulator mutants (*bcr1*Δ/Δ, *efg1*Δ/Δ, and *efg1*Δ/Δ *cph1*Δ/Δ) or tetracycline-regulatable strains (TNRG1) that demonstrated decreased immunopathology *in vivo*. Strains DAY185 and TT21 served as appropriate controls. Mice underwent vaginal lavage at day 3 postinoculation, and those same mice were lavaged again at day 7. Lavage fluids were assessed for fungal burdens by standard microbiological plating (medians) (A), PMNs by Pap staining (means ± SEM) (the horizontal dashed line denotes the high-responder cutoff) (B), S100A8 protein by ELISA (means ± SEM) (C), IL-1β by ELISA (means ± SEM) (D), and tissue damage by LDH activity (means ± SEM) (E). All data are cumulative data from two independent experiments ($n = 4$ per group; 8 mice total). Statistical analysis for significance was accomplished by using a one-way ANOVA with Dunnett's posttest to compare transcriptional regulator mutants to DAY185 and an unpaired Student *t* test to compare TNRG1 to TT21 at day 3 and day 7, respectively. Significance is denoted as follows: ∗ or #, $P < 0.05$; ∗∗ or ##, $P < 0.01$; ∗∗∗ or ###, $P < 0.001$. A paired Student *t* test was also used to compare values between day 3 and day 7 within infection groups, although these results were not statistically significant.

that a threshold for activation or duration of epithelial cell signaling exists, such that as more yeast cells undergo morphogenesis, resulting in increased expression of hyphal pathogen-associated molecular patterns (PAMPs), amplified levels of immune responses become evident. Still other mechanisms of mucosal immunoregulation, including damage-induced inflammasome activation, may explain the observable phenotypes (39). The correlation of IL-1β secretion with tissue damage (Fig. 1F) and fungal morphogenetic dependence (Fig. 3 and 4) to elicit a response suggests a potentially important role for inflammasome signaling in the vaginal mucosa during vaginitis (40, 41). However, comprehensive *in vivo* studies using mice deficient in inflammasome components and IL-1 signaling are required to rigorously test this hypothesis.

Systemic immunodepletion of PMNs was utilized to examine their role in contributing to vaginitis immunopathology. Similar to studies conducted by Black et al., reduction of PMNs had no effect on vaginal fungal burden, suggesting a nonprotective role during vaginitis (Fig. 2A) (26). Interestingly, PMN depletion had profound effects on the reduction of both vaginal S100A8 and IL-1β levels (Fig. 2C and D), suggesting that a majority of the innate immune signaling may be attributed directly to recruited neutrophils, as PMNs have been shown to make both of these inflammatory stimuli (9, 42). Despite these reductions, it is important to point out that significant amounts of S100A8 and IL-1β were still generated in PMN-depleted animals, suggesting another source for these inflammatory mediators (Fig. 2F). In support of this, Yano et al. isolated murine vaginal epithelial cells and demonstrated that S100A8/9 could be induced *in vitro* upon inoculation with *C. albicans* (8). Histological evidence of S100A8/9 expression in the vaginal epithelium further confirmed these findings. Similarly, several *in vitro* models using human vaginal

epithelial cells have also demonstrated increased IL-1 secretion during infection with *C. albicans* (15, 43, 44). Therefore, compensatory activity from IL-1β production may also explain the recruitment of PMNs to the vagina during murine vaginitis in the absence of a functional S100A8/9 complex (52). Importantly, LDH activity (Fig. 2E) was not different among control and PMN-depleted animals, supporting the idea that vaginal tissue damage is not mediated by PMN infiltration but rather by active fungal infection. Therefore, therapeutic attempts to reduce PMN-mediated symptomatologies during vaginitis are unlikely to enhance infection and may offer promising potential as novel immunotherapeutic approaches.

The yeast-to-hypha morphogenetic switch in *C. albicans* is often described as the major virulence factor of this fungal pathogen, as hypha formation is associated with elevated secretion of hydrolytic enzymes, direct invasion of tissue layers, and increased adherence to host surfaces (45). Because tissue damage and the presence of fungi are correlated with increases in innate immune signaling during vaginitis, it begged the question of whether defects in fungal morphogenesis affect host immunopathology. The switch from yeast to hypha is not a trivial process but rather involves a highly intricate network of environmental cues linked to fungal regulatory genes (46). The major morphogenetic pathways in *C. albicans* include PKA activation via Efg1, Cph1 signaling via MAPK cascades, the pH-regulated RIM101 pathway, Czf1 activation by contact dependence, and osmotic stress responses via the HOG pathway. There are likely other pathways that have yet to be completely defined, and the interdependent signaling mechanisms utilized by known pathways further cloud the complete picture of morphogenetic regulation. However, innate immune responses to *C. albicans* strains defective in their ability to undergo morphogenesis were clearly suppressed (Fig. 3). Interestingly, deletion of *CPH1* had no effect on fungal morphology or immunopathology during vaginitis. This may possibly be due to the fact that *CPH1* mutants fail to make hyphae only under strict conditions, such as metabolic stress; it is unlikely that *C. albicans* is starved in a nutrient-dense milieu of host epithelial tissue during vaginal infection. Based on our findings, it appears that the Efg1 pathway is crucial for initiating and antagonizing the immune response during vaginitis, evidenced by reduced immune responses that were recovered with the reconstituted strain. Importantly, forcing cells to maintain either yeast (TNRG1) or hypha (TUME6) morphologies by overexpressing key transcription factors also yielded similar results (Fig. 4). Interestingly, inoculation with TUME6 did not lead to higher levels of inflammatory markers, as would be predicted with this constitutively filamentous strain. Perhaps, the response is already maximally engaged with the isogenic control strain, or forcing morphogenesis independent of environmental sensing offsets potential enhanced virulence. This complementary approach further supports a critical role for the contribution of morphogenetic regulation to vaginitis immunopathology.

A previous study by Sobel et al. using a rat model of vaginitis identified germ tube formation as an important virulence determinant (16). Intravaginal inoculation with a natural variant of *C. albicans* unable to filament at 37°C or in serum led to lower fungal burdens and increased rates of spontaneous clearance. Unfortunately, the specific genetic defects in morphogenesis of this strain remain undetermined, and therefore, comparisons to nonisogenic control strains are difficult. Nevertheless, the results obtained by Sobel et al. are directly opposed to the increased fungal burdens observed in these studies when using strains (*efg1*Δ/Δ, *efg1*Δ/Δ *cph1*Δ/Δ, and TNRG1) that are unable to undergo morphogenesis (Fig. 3 and 4). It was initially thought that elevated levels of fungal recovery in the lavage fluid might be due to a decreased adhesion of these strains to the vaginal mucosa. However, quantitative fungal burdens obtained from vaginal tissue homogenates revealed the same colonization patterns (data not shown), suggesting that potential adherence defects alone do not significantly influence fungal burdens recovered during vaginal lavage. Smaller size, unicellularity, and potentially increased rates of cell turnover on the vaginal epithelium of yeast cells may explain differences in fungal burdens observed between nongerminating and germinating strains. Assessment of fungal burden by quantifying fungal genomes by PCR amplification may be useful in determining more precise quantitative differences between morphogenetically competent and deficient strains. However, even if fungal burdens were found to be similar by genome quantification, strains unable to undergo morphogenesis would still demonstrate an inability to elicit robust immune responses *in vivo*. Furthermore, measurement of fungal burden *in vivo* by quantitative plate counting remains the gold-standard method, and our results reflect the expected outcomes using these strains.

Efg1 and Bcr1 have been termed master regulators of *C. albicans* pathogenesis, because they are directly associated with morphogenesis and expression of hypha-associated virulence characteristics (47, 48). It has long been debated whether merely the presence of hyphae or these hypha-associated factors are involved in immune activation during candidiasis. *In vitro* infection of reconstituted human vaginal epithelium demonstrated that distinct immunological pathways exist for discriminating yeast from hyphae (15). Findings from the current study revealed that although the *bcr1*Δ/Δ strain undergoes morphogenesis and forms hyphae that are morphologically similar to those formed by WT *C. albicans*, it demonstrates defects in the ability to elicit similarly robust immune responses *in vivo*. The seemingly intermediate phenotype observed for the *bcr1*Δ/Δ strain to elicit tissue damage was also reported previously by Dwivedi et al. using a mouse model of oral candidiasis (49). In that study, analysis of tissue fungal burdens revealed that the *bcr1*Δ/Δ strain was found to colonize the tongue surface less efficiently than its WT or reconstituted strain. Interestingly, in our study, we also observed generally lower fungal burdens recovered from mice inoculated with the *bcr1*Δ/Δ strain, but the results were not statistically significant (Fig. 3 and 6). Although not as severe as the loss of *EFG1* expression, ablation of Bcr1-mediated signaling noticeably results in reduced vaginitis immunopathology. Based on these results, we conclude that simply the presence of hyphae is insufficient to stimulate an immune response comparable to that of the WT and that the aggregate regulation of hypha-associated virulence factors (perhaps those regulated by Bcr-1) may be required. Therefore, the use of morphogenesis (the act of the yeast-to-hypha transition) may be somewhat of a misnomer in relation to disease pathogenesis, and we propose the use of morphogenetic response (cumulative phenotype of hypha transition) as more appropriate terminology.

One major consequence of the loss of *BCR1* or *EFG1* expression is the impaired ability of *C. albicans* to form biofilms (14). Biofilms in *C. albicans* confer fungal resistance to neutrophils, antifungal agents, and mechanical stressors, presumably through the elaboration of a polysaccharide-rich extracellular matrix (50).

While it is tantalizing to speculate that biofilm formation represents a trigger for recognition by the innate immune system, the intimate linkage of biofilm regulation with fungal morphogenesis makes interpretation extremely difficult. However, it is conceivable that a biofilm-specific moiety and not morphogenesis *per se* leads to PMN recruitment and eventual symptomatology during vaginal infection. Generation of strains defective in biofilm-associated factors (e.g., extracellular matrix production) but not morphogenetic regulation may answer these important questions. Overall, our data strongly support the long-standing belief that asymptomatic colonization of mucosal surfaces is attributed to yeast forms of *C. albicans*. However, transition to hyphal forms, accompanied by assembly of characteristic biofilm architecture, triggers significant tissue damage and the activation of host innate immune pathways leading to elevated levels of production of inflammatory markers, neutrophil recruitment, and, ultimately, symptomatic infection.

In conclusion, we have demonstrated that the immunopathology associated with *Candida* vaginitis is positively correlated with tissue damage, S100 alarmin production, and IL-1β secretion *in vivo*. Hence, IL-1β may be an alternative inflammatory marker equally capable of initiating or sustaining the PMN response. It was also demonstrated that the inflammatory responses observed during murine vaginitis are dependent primarily on the Efg1-dependent pathway of fungal morphogenesis. However, loss of Bcr1-mediated signaling in *C. albicans* also results in reduced immunopathology *in vivo* albeit not as dramatically. Despite revealing these important findings, a number of imperative questions still remain, including whether the presence of hyphae, induced damage, or both are sufficient for characteristic inflammatory responses and which hypha-associated factors are required for disease pathology. Since fungal phenotypes that show reduced immunopathology *in vivo* have now been uncovered, experiments designed to reconstitute the expression of various candidate virulence determinants in these strains may elucidate the role of individual adhesins or secreted factors in activating host innate immunity pathways during vaginitis. Collectively, results from this study contribute to a more comprehensive understanding of the immunopathogenesis of *Candida* vaginitis and may likely explain the ability of *C. albicans* to colonize the vagina as both an asymptomatic commensal (yeast) and an inflammatory pathogen (morphological transition to hyphae). Ultimately, cell signaling pathways involved in the yeast-to-hypha transition may serve as novel drug targets in controlling associated pathologies during *Candida* vaginitis.

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